REMARKS

Applicants have amended claim 1 to make explicit that the claimed method is directed to the absolute quantification of the amount of the nucleic acids. Support for the amendments can be found throughout the specification, for example, in paragraph [007], lines 4-6. Claims 4-9 remained cancelled. New claim 15 has been added to further specify that a preferred method for absolute quantification of the nucleic acids consists essentially of the steps of a) through e). Support for this amendment can be found throughout the specification, for example, in paragraphs [0027]-[0028] as well as in the Example.

Accordingly, no new matter is introduced by the amendments and their entry is respectfully requested.

Applicants now turn to the specific rejections.

The Examiner rejected claims 1-3 and 10-13 under 35 U.S.C. 103(a) as allegedly being unpatentable over Becker et al. (Nucleic Acid Research, 1989, vol. 17, no. 22, pages 9437-9446) in view of Amexis et al. (PNAS. October 2001, vol. 98, no. 21, pages 12097-12102) and Ross et al. (BioTechniques, September 2000, vol. 29, pages 620-629). Applicants respectfully disagree and submit that the rejection be withdrawn for the following reasons.

Applicants have amended claim 1 as described, *supra*. Applicants also submit herewith a Declaration by Dr. Charles Cantor ("Declaration"), an expert in this field, setting forth reasons why a skilled artisan would not have considered the present method by reading Becker in view of Amexis and Ross.

The present claims are directed to **absolute quantification** of **multiple target** nucleic acids with **multiple internal standards** using **single base primer extension reactions** and mass spectrometric detection methods.

Becker quantified a single nucleic acid using a single standard that differed by one nucleotide from the target so that a restriction enzyme would digest one of the amplified nucleic acids. [See also Par. 7 of Declaration]. Although Becker discussed the possibility of absolute quantification, Becker **required an extra step of diluting the PCR reaction mixture** prior to the last PCR cycle in the amplification step in order to be quantitative and determine an absolute amount of a single target nucleic acid. [Par. 8 of Declaration]

Becker especially emphasized the importance of this diluting step for the purpose of comparing the amount of signal determined from the target nucleic acid against the amount of signal determined from the standard nucleic acids, as cited:

> "Using a mixture of authentic (endogenous = en) and mutated (exogenous = ex) *in vitro* RNA^{4CL} transcripts we could show that the ratio of signal intensities of the detected bands represented the ratio of RNA amounts present in the beginning. However, *it was crucial to dilute the sample before the last PCR cycle. Otherwise, the upper band (en) was consistently over-represented.*" See Becker, page 9440, paragraph 2, lines 1-6; emphasis added.

Moreover, Becker also described that this **dilution step is necessary for absolute quantification** of a nucleic acid in order to avoid the problem of "heterodimeric DNA" phenomenon after certain cycle numbers of PCR amplification. See Becker, e.g., page 9440, paragraph 2, lines 7-12; page 9443, paragraph 2, lines 1-5. Therefore, without the dilution step, the result of quantification in Becker would not have been accurate, and the quantification of an absolute amount of target would have been greatly compromised. [Par. 9 of Declaration].

In contrast, Applicants have explicitly addressed in the specification that the absolute quantification method of the claimed invention needs virtually no optimization for PCR amplification. Hence Applicants' absolute quantification method does not need the dilution step in any of the PCR cycles. Applicants' absolute quantification method is also independent of PCR cycle numbers. [Par. 10 of Declaration].

Moreover, the heterodimeric DNA problem confronting Becker means that the accuracy of each assay will be different and accounting for this difference requires a correction factor that will be different for each assay (**every target**). In contrast, the claimed method needs no adjustment specific to each target; and the claimed method discloses **absolute quantification** of **multiple target** nucleic acids in the **same reaction** without any optimization. Accordingly, Becker does not teach or suggest a method that would be useful as an absolute quantification method and/or allow at least two or more targets to be analyzed simultaneously. [Par. 11 of Declaration].

Amexis and Ross do not cure Becker's deficiency since neither Amexis nor Ross even mention that their methods can be applied for absolute quantification. [Par. 13 of Declaration].

Amexis quantified the **relative levels** of two virus variants in one reaction through PCR and MassArray system. See Amexis, e.g., page 12100, 1st column, last paragraph. Comparing the **relative amount** of allelic variants does not allow absolute quantification of nucleic acid species in the reaction. Additionally, because Amexis evaluated relative amounts of allelic products already in the sample, Amexis did not add an external standard. [Par. 14 of Declaration].

Ross also quantified the *relative levels* of pooled allelic variants and therefore, for the same reason as Amexis, does not describe how absolute quantification could be achieved. See Ross, e.g., page 624, 1st column, 1st and 2nd paragraphs. Also Ross did not use an external standard. [Par. 15 of Declaration].

Moreover, both Amexis and Ross compared the relative amount of allelic variants. Therefore, targets analyzed by the methods described by Amexis and Ross are limited to those targets that comprise an allele (e.g., polymorphism). In contrast, the methods that Applicants described are polymorphism-independent, thus allowing for the absolute quantification of a wider range of targets (e.g., gene sequences that do not contain a polymorphism). [Par. 16 of Declaration].

By citing Ross, the Examiner also contended that it would have been obvious to combine mutation analysis of Becker with **single base primer extension reactions** and MALDI-TOF analysis in Ross to arrive at Applicants' claimed invention.

It is well known and also stated in Ross that single base extension, like the one used in the presently claimed methods, produces mass differences between 9 and 40 Da. [Par. 17 of Declaration]. However, Ross specifically states that "baseline resolution between alleles differing by 16 Daltons (Da) or less may not be observed." See Ross, page 622, 1st column. [Par. 18 of Declaration]. Ross also states that "area measurement of a low-intensity extension produces within 40 Da of another allele may be confounded by trace cation…adducts onto the lower mass allele." See Ross, page 622, 1st column. [Par. 19 of Declaration]

Therefore, Ross teaches that they made sure that all primer extensions resulted in mass differences between 300-400 Da. See Ross, Page 622, 1st column. Ross specifically taught that "two related strategies were selected by which a molecular weight separation of about

300-400 Da between allele products of a given locus could be achieved during the primer extension assay." See Ross, page 622, 3rd paragraph, lines 1-6. Ross expected a clear separation of 300-400 Da between alleles and extension products for reliable peak detection and reliable quantification of nucleic acids. For example, Ross used a strategy of terminating the variants of the nucleic acid by one (wild-type) and two (mutant) bases, thus enhancing the mass difference; and another strategy they terminated the variants of the nucleic acid by one base (wild-type) and a fluorescently labeled base (mutant). Neither one of the modified primer extension strategies of Ross, is the same as the single-base primer extension method of the present invention. [Par. 20 of Declaration]. The single base extension like the one Applicants used, does not necessarily produce mass differences of 300-400 Da. [Par. 21 of Declaration].

Therefore, **Ross essentially teaches against or away** from the method Applicants found to be most effective for absolute quantification purposes because Ross essentially teaches that the method Applicants use cannot differentiate nucleic acids well enough in MALDI-TOF analysis. [Par. 22 of Declaration]

In view of the above, Applicants respectfully submit that one of ordinary skill in the art would not have expected that combination of the mutation analysis of Becker with MALDI-TOF analysis **using a single base extension** could be used to provide accurate quantitative measurements of the **absolute amount** of nucleic acids in a sample. [Par. 23 of Declaration]

Even if one were to combine the references, one would be expected to use dilution of PCR mixture before last PCR cycle to obtain a sample that might allow absolute quantification and **one would have not used a single base extension** but an extension reaction that would have resulted in differences between 300-400 Da in molecular weight of the control and the allele one wishes to quantify. [Par. 24 of Declaration]

Moreover, one would have been skeptical about quantifying after the dilution step because it could have been considered to lead to a very low amount of sample that would have lowered the peak intensity, sacrificed the signal to noise level and returned an unreliable quantification result when using MALDI-TOF. Therefore, one would not have expected the combination of Becker with Ross and/or Amexis to work. [Par. 25 of Declaration]

Additionally, as explained by Dr. Cantor, if it had been obvious to use Becker to design an absolute quantification method using mass spectrometry, which has been generally known as an analysis tool since at least the mid 1980's with commercial instruments introduced in the early 1990s, it would not have taken over 10 years from the publication of Becker to develop such a method. [Par. 12 of Declaration].

In contrast, as already presented in the previous response, Applicants surprisingly discovered that Applicants' method can accurately **quantify the absolute amount** of multiple target sequences with multiple internal standards in the same reaction (e.g., triplex targets). Applicants found that the extension products were clearly separated in the mass spectrum with very strong signal to noise level. In particular, the mass differences between several extension products were very small. For example, mass difference between glut3-S and glut3-A was only about 20-25 Da, yet, contrary to what Ross described, Applicants found that the two peaks were clearly separated with strong peak intensities. See September 10, 2007 Response, page 6, last paragraph to page 7, paragraph 2 and Exhibit A. These absolute quantification results by multiplex reactions agreed well with those from uniplex reactions. Moreover, Applicants found that the same method can be used to quantify at least about 20 targets in one multiplex reaction. [Par. 26 of Declaration]

Accordingly, contrary to the Examiner's argument, at the time of the invention, *absolute quantification* of multiple nucleic acids using mass spectrometric detection and single base extension reactions in the same reaction was not something scientists performed or would have expected to succeed. One skilled in the art would not have been motivated to use multiple internal standards with multiplex target nucleic acids for absolute quantification of multiplex without diluting the amplified mixtures, and one would not have been motivated to subsequently use mass spectrometric analysis combined with single-base primer extension for absolute quantification of multiple nucleic acids in the same reaction, particularly when the multiple nucleic acids differentiating only by *small mass differences*. [Par. 27 of Declaration]

Therefore, Applicants submit that Becker does not teach or suggest absolute quantification of multiple target nucleic acids with multiple external standards using a single base extension with mass spectrometry detections. Amexis and Ross do not overcome this

deficiency. In light of the amendments and the arguments presented above, Applicants respectfully submit that the rejection under 35 U.S.C. 103(a) over Becker in view of Amexis and Ross should be withdrawn.

The Examiner also provisionally rejected claims 1-3 and 10-13 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-12 of copending Application No. 10/589,709. Applicants request that the provisional rejections be held in abeyance until the claims are otherwise in condition of allowance.

In view of the foregoing, Applicants respectfully submit that all claims are in condition for allowance.

Early and favorable action is respectfully requested. Examiner is encouraged to contact the undersigned attorney should there be additional questions regarding the application.

In the event that any additional fees are required, the Commissioner is hereby authorized to charge Nixon Peabody LLP deposit account No. 50-0850.

Date: February 26, 2010

Respectfully submitted,

/Leena H. Karttunen/

Customer No.: 50607

Ronald I. Eisenstein (Reg. No. 30,628)
Leena H. Karttunen (Reg. No. 60,335)
(617) 345-6054 / 1367